(12) UK Patent Application (19) GB (11) 2 306 485 (13) A

(43) Date of A Publication 07.05.1997

(21) Application No 9622819.2

(22) Date of Filing 01.11.1996

(30) Priority Data
(31) PCT/GB95/02588 (32) 02.11.1995 (33) WO
9522407 01.11.1995 GB

(71) Applicant(s)
British Gas plc

(Incorporated in the United Kingdom)

Rivermill House, 152 Grosvenor Road, LONDON, SW1V 3JL, United Kingdom

(72) Inventor(s)
Luet-Lok Wong
Sabine Lahja Flitsch
Darren Paul Nickerson
Alwyn James Hart

(51) INT CL$^6$
C12N 9/02 15/53 // ( C12N 15/53 C12R 1:19 )

(52) UK CL (Edition O )
C3H HB7M H650 H685
C6Y Y125 Y153 Y501 Y503
U1S S1289 S1305 S1333 S1508

(56) Documents Cited
GB 2294692 A
J.Biol.Chem. 1988,263(35),18842-18849
J.Amer.Chem.Soc. 1989,111,2715-2717 J.Biol.Chem. 1990,265(10),5361-5363

(58) Field of Search
UK CL (Edition O ) C3H HB7M
INT CL$^6$ C12N 9/02 15/53
ONLINE: WPI,CLAIMS,DIALOG/BIOTECH

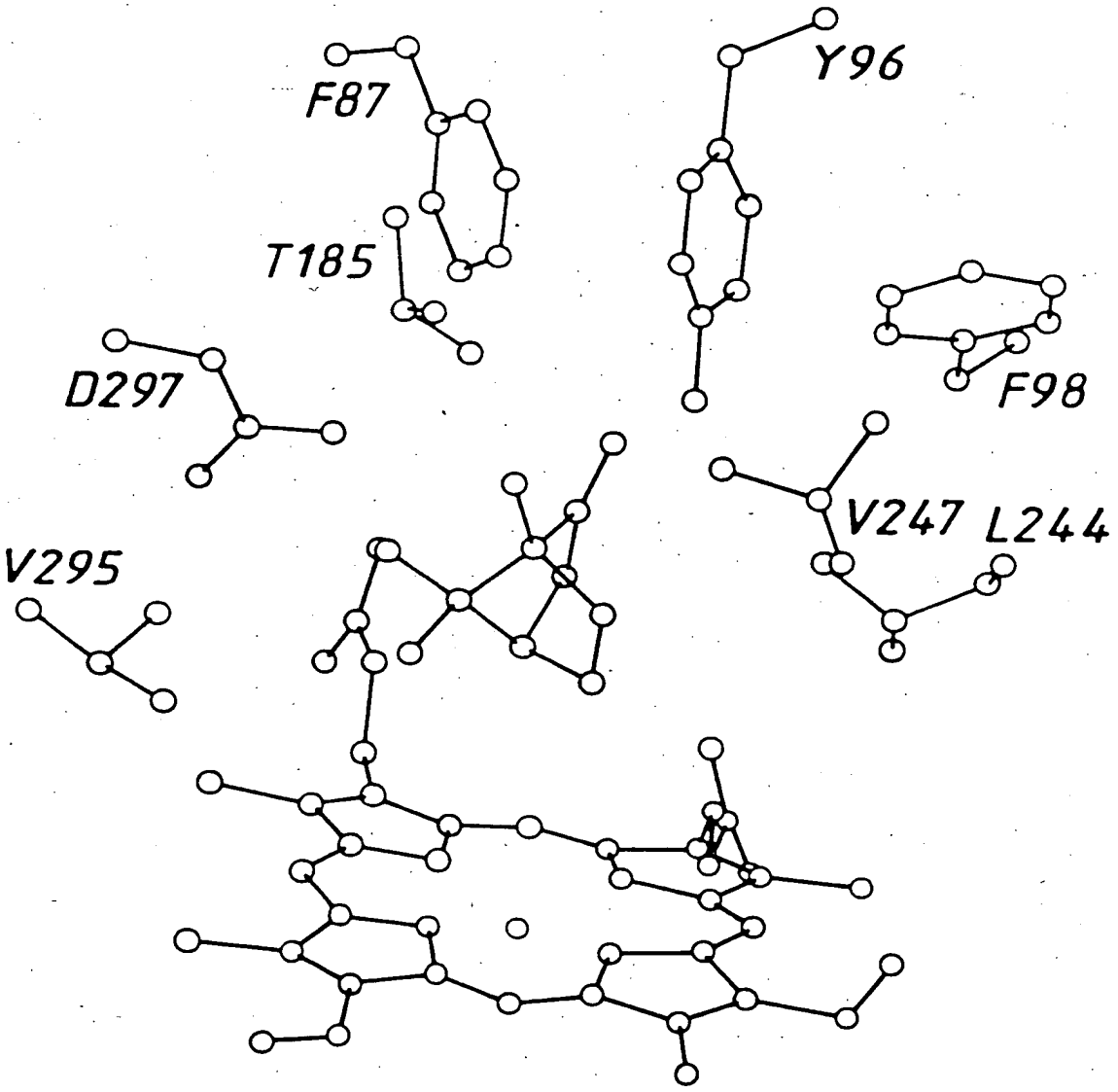
(74) Agent and/or Address for Service
David J Morgan
British Gas plc, Intellectual Property Department, 100 Thames Valley Park Drive, READING, Berkshire, RG6 1PT, United Kingdom

(54) Mutants of mono-oxygenase cytochrome P-450cam.

(57) A mutant of mono-oxygenase cytochrome P-450cam in which cysteine, at position 334, is deleted therefrom, or replaced by a different amino acid, is described. Tyrosine(96) may also be substituted by a different amino acid. The amino acid may be selected from alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan and valine, and, for cysteine(334), tyrosine. Preferred substitutions of amino acids may also occur at at least one of the positions 87, 98, 101, 185, 193, 244, 247, 295, 297, 395 and 396.

GB 2 306 485 A

FIG.1.



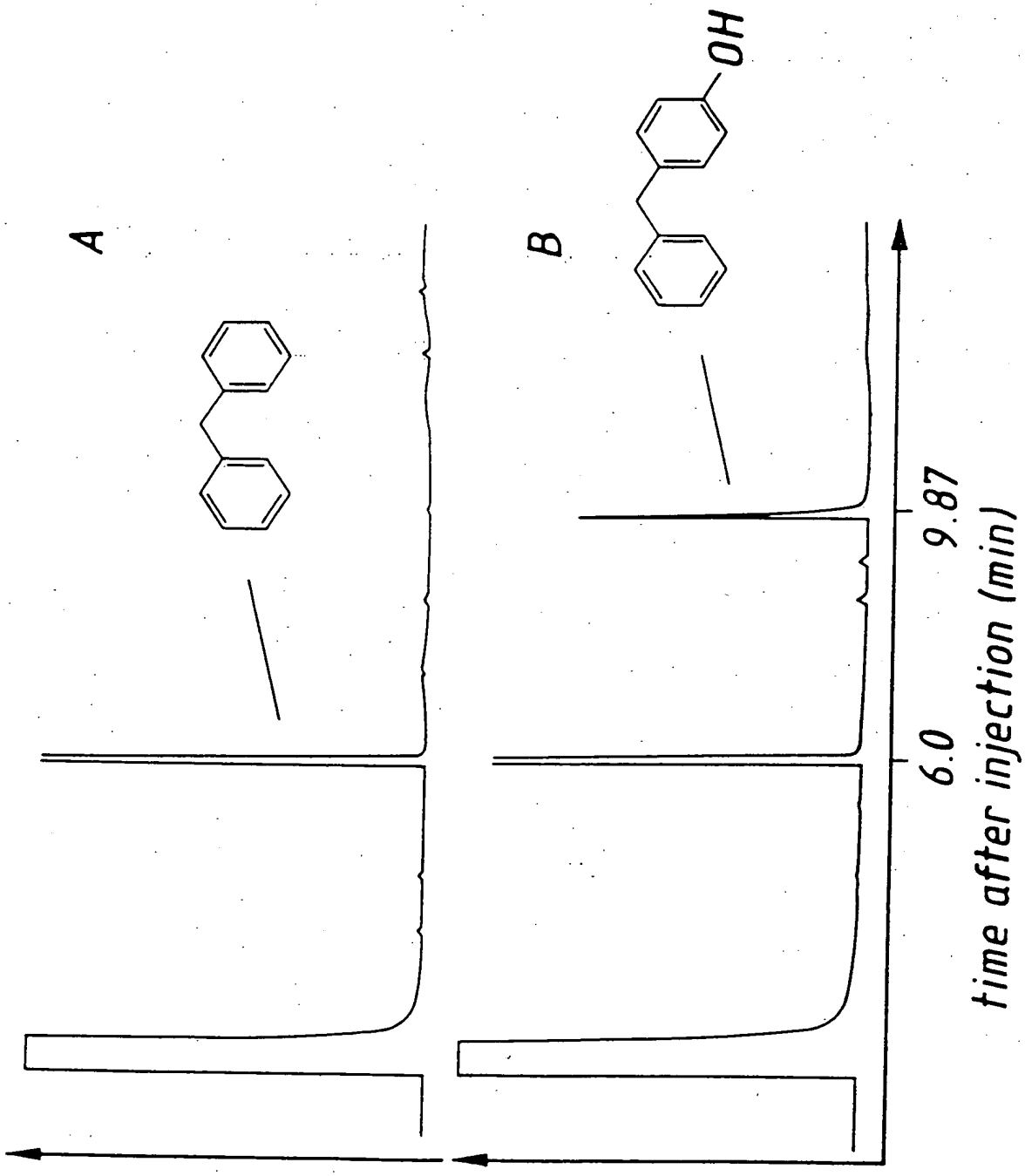



FIG.2.

GRC 4269

# ENZYME MUTANT

The present invention relates to a mutant of the mono-oxygenase cytochrome P-450cam.

Mono-oxygenases catalyse the selective oxidation of activated and unactivated carbon-hydrogen bonds using oxygen[1], and are therefore of great interest for potential use in organic synthesis. However, progress in this area has been hampered by the difficulty in isolating sufficient quantities of the mono-oxygenase enzyme and/or the associated electron-transfer proteins. Despite the availability of amino acid sequences of more than 150 different cytochrome P-450 mono-oxygenases, to date structural date of only three are available[2,3,4], and few have been successfully over-expressed in bacterial systems[5].

One cytochrome P-450 mono-oxygenase, which is soluble and can be expressed in sufficient quantities, is the highly specific P-450cam from P. putida which catalyses the regio- and stereo-selective hydroxylation of camphor to 5-exo-hydroxycamphor[6]. The high resolution crystal structure of P-450cam has been determined[2], and since the mechanism of action of this bacterial enzyme is believed to be very similar to that of its mammalian counterparts, it has been used as a framework on which structural models of mammalian enzymes are based.

The nucleotide sequence and corresponding amino acid sequence of P-450cam have been described[5,7]. The location of an active site of the enzyme is known and structure-function relationships have been investigated[8,9]. Mutants of P-450cam have been described at the 101 and 185 and 247 and 295 positions[9,10,11] and at the 87 position[12]. A mutant in which tyrosine 96 (Y96) has been changed to phenylalanine 96 (the Y96F mutant) has been described[11,13,14,15]. But in all cases the papers report effects of the mutations on the oxidation reactions of molecules which had previously been shown to be substrates for the wild-type enzyme. There is no teaching of how mutations might be used to provide biocatalysts for oxidation of different, novel substrates.

In an attempt to develop new biocatalysts, we have initiated a project which aims to redesign P-450cam, such that it is able more effectively to carry out specific oxidations of organic molecules whether or not these are substrates for the wild-type protein.

The three dimensional structure of P-450cam shows the active site to provide close van der Waals contacts with the hydrophobic groups of camphor as shown in Figure 1. Of particular significance are the contacts between camphor and the side chains of leucine 244, valine 247 and valine 295. Three aromatic residues (Y96, F87 and F98) are grouped together and line the substrate binding pocket, with a hydrogen bond between tyrosine 96 and the camphor carbonyl oxygen maintaining the

substrate in the correct orientation to ensure the regio- and stereo- specificity of the reaction.

Lipscomb and co-workers[16] demonstrated in 1978 that wild-type P-450cam showed a propensity to dimerise, but they also reported that the catalytic activity of the monomer and dimer towards camphor oxidation were indistinguishable. Since the dimerisation reaction could be reversed by thiol reducing agents, they concluded that it occurred by intermolecular cysteine disulphide (S-S) bond formation. They were unable to determine whether dimerisation involved more than one cysteine per P-450cam molecule. Nor were they able to identify the key cysteine residue(s) involved in this reaction because neither the amino acid sequence nor crystal structure of P-450cam were known at the time.

We used molecular modelling to investigate the likely effects of points mutations to the three aromatic residues (Y96, F87, F98) in the active site pocket. We noted that replacement of any of these aromatic residues with a smaller, hydrophobic non-aromatic side-chain could provide an "aromatic pocket" which could be used to bind more hydrophobic substrates. The program GRID[17] was used to calculate an energy of interaction between an aromatic probe and possible mutants of cytochrome P-450cam where these residues were changed to alanine (F87A, Y96A and F98A). The results were then examined graphically using the molecular modelling package Quanta[18].

The mutant F98A appeared to have the strongest binding interaction within the active site cavity accessible to the aromatic probe, with that of Y96A being slightly smaller, and that of F87A being substantially less. It was decided in the first instance to mutate tyrosine 96 to alanine as it is more central to the binding pocket, whereas phenylalanine 98 is in a groove to one side. Also, removal of tyrosine 96 should decrease the specificity of the enzyme towards camphor due to the loss of hydrogen bonding to the substrate.

According to one aspect of the present invention a mutant of the mono-oxygenase cytochrome p-450cam is provided in which the cysteine residue at position 334 is removed.

Preferably the removal is by the substitution of another amino acid except cysteine for the cysteine residue.

Alternatively the removal is by the deletion of the entire cysteine 344 residue from the enzyme.

Suitably the tyrosine residue at position 96 in the mutant is replaced by the residue of any amino acid except tyrosine.

Conveniently the amino acid is selected from any one of the following: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan, tyrosine and valine except that in the

case of the cysteine residue at position 334, the amino acid is not cysteine and in the case of the tyrosine residue at position 96 the amino acid is not tyrosine.

Preferably the amino acid residue at one or more of the positions 87, 98, 101, 185, 193, 244, 247, 295, 297, 395 and 396 is replaced by another amino acid residue.

We examined the structure of P-450cam generated from the published crystallographic atomic co-ordinates using the modelling programme Quanta. We determined that there are five cysteines near the surface of P-450cam (cysteines 58, 85, 136, 148, 334) which might participate in intermolecular disulphide bond formation leading to protein dimerisation. We carried out sit-directed mutagenesis to substitute each of these cysteines to alanine, thus generating five Cys - Ala surface mutants.

The extent of protein dimerisation in the wild-type P-450cam and the five surface Cys - Ala mutants were investigated, The presence of dimer was detected by both anion exchange fast protein liquid chromatography on a Resource Q column (Pharmacia) and gel filtration size exclusion chromatography on a Superose 12 column (Pharmacia) in the wild-type P-450cam and the C58A, C85A, C136A and C148A mutants. On the other hand, no dimer was detected, even at high concentrations (0.1mM range), for the C334A mutant (see data in Figure 2). We concluded that wild-type P-450cam underwent dimerisation by intermolecular S-S

disulphide bond formation between the surface cysteine 334 on two protein molecules.

The C334A mutation has the obvious benefit of removing unwanted protein dimerisation, thus ensuring the presence of a single species in solution at all times. In addition, we noted a completely unexpected benefit of this mutation. Like all proteins, wild-type P-450cam shows aggregation upon standing. The reasons why proteins aggregate are not clear, but the P-450cam aggregates are insoluble and catalytically inactive. The wild-type and C58A, C85A, C136A and C148A mutants all showed dimerisation as well as aggregation upon storage at 4°C, and even in 50% glycerol solutions at -20°C. Aggregation will also occur during turnover, especially at the higher P-450cam concentrations required in any economically viable industrial application in, for example, synthesis of organic molecules. The C334A mutant did not show any evidence of aggregation even at mM concentrations at room temperature over a period of three days. Thus, the C334A mutation has beneficial effects in protein handling, storage, and increased catalyst lifetime.

We believe the mutation at position 96 to be the key which enables the mutant enzymes to catalyse the oxidation of a relatively wide range of organic substrates. Other amino acids adjacent to the active site of the enzyme may also be mutated in order to change the shape and specificity of the active site. These other amino acids include those at positions 87, 98, 101, 185, 193, 244, 247, 295, 297, 395 and 396. It is envisaged that

the amino acid at one or more of these positions may be replaced by: a small hydrophobic amino acid so as to enlarge the active site; or a large hydrophobic amino acid so as to reduce the size of the active site; or by an amino acid having an aromatic ring to interact with a corresponding aromatic ring of a substrate.

Regarding the oxidation reactions, the conditions are described in the literature references attached. The enzyme system typically includes putidaredoxin and putidaredoxin reductase together with NADH as co-factors in addition to the mutant enzyme. The example of cyclohexylbenzene oxidation is described in the experimental section below. Various classes of organic compounds are envisaged and described below. We note that the wild-type P-450cam is active towards the oxidation of a number of molecules included in the following sections. However, in all cases the mutant P-450cam proteins show much higher turnover activities.

i) The organic compound is an aromatic compound, either a hydrocarbon or a compound used under conditions in which it does not inactivate or denature the enzyme. Since the mutation has been effected with a view to creating an aromatic-binding pocket in the active site of the enzyme, the mutant enzyme is capable of catalysing the oxidation of a wide variety of aromatic compounds. Oxidation of example aromatic and polyaromatic compounds is demonstrated in the experimental section below and is believed very surprising given that the wild-type enzyme has been reported to

catalyse the oxidation of only members of the camphor family and shows low activity towards a few other molecules such as styrene[19], ethylbenzene[9,10], a tetralone derivative[20], and nicotine[21].

ii) The organic compound may be a hydrocarbon, e.g. aliphatic or alicyclic, carrying a functional group (see Scheme 1). An aromatic protecting group is attached to the functional group prior to the oxidation reaction and removed from the functional group after the oxidation reaction. A suitable aromatic group is a benzyl group. The protecting group serves two purposes: firstly it makes the substrate more hydrophobic and hence increases binding to the hydrophobic enzyme pocket; secondly it may help to hold the substrate in place at the active site. Thus, with the correct aromatic protection group, both regio- and stereo-selective hydroxylation of the substrate may be achieved. Examples of monofunctionalised hydrocarbons are cyclohexyl, cyclopentyl and alkyl derivatives (Scheme 1). The oxidation products of these compounds are valuable starting materials for organic synthesis, particularly when produced in a homochiral form. A range of aromatic protecting groups are envisaged, e.g. benzyl or naphthyl ethers and benzoyl ethers and amides (Scheme 1). Of interest are also benzoxazole groups as carboxyl protecting groups and N-benzyl oxazolidine groups as aldehyde protecting groups. Both can be easily cleaved after the enzymatic oxidation

and have previously been described in the literature for the microbial oxidations of aldehydes and acids[22].

iii) The organic compound is a C4 to C12 aliphatic or alicyclic hydrocarbon. Oxidation of cyclohexane and linear and branched hydrocarbons is demonstrated in the experimental section below. We have found that wild-type P-450cam is also capable of oxidising these molecules, but the activities are low and in all cases the mutants show substantially higher activities.

iv) The organic compound is a halogenated aliphatic or alicyclic hydrocarbon. Oxidation of lindane (hexachlorocyclohexane) is also describe below.

Mutants were constructed in which active site substitutions were combined with the surface mutation of cysteine at position 334 to alanine and contained alanine, leucine, valine, or phenylalanine instead of tyrosine at position 96 (Y96). Lastly several active site mutations and the surface mutation were combined to constitute mutant enzymes with multiple mutations. The genes encoding cytochrome P-450cam, and its natural electron-transfer partners putidaredoxin and putidaredoxin reductase, were amplified from the total cellular DNA of P. Putida using the polymerise chain reaction (PCR). The expression vector/E. coli host combinations employed were pRH1091[23] in strain JM109 for P-450cam, pUC 118 in strain JM109 for putidaredoxin, and pGL W11 in strain DH5 for putidaredoxin

reductase. Oligonucleotide-directed site-specific mutagenesis was carried out using an M13 mp 19 subclone by the method of Zoller and Smith[24], and mutant selection was by the method of Kunkel[25].

Binding of potential substrates was investigated by spectroscopic methods. The wild-type enzyme in the absence of substrate is in the 6-co-ordinated, low-spin form with a weakly bound water occupying the sixth co-ordination site, and shows a characteristic Soret maximum at 418 nm. Binding of camphor and the substrate analogues adamantanone, adamantane and norbornane fully converted the haem to the 5-co-ordinated, high-spin form which has a characteristic Soret band at 392 nm. This haem spin-state shift is accompanied by an increase in the haem reduction potential which enables the physiological electron-transfer partner putidaredoxin to reduce P-450cam and initiate the catalytic hydroxylation cycle[26]. The haem spin state shift is thus a qualitative indication of the likelihood of molecules shown in Tables 1 and 2 being oxidised by the wild-type and mutant P-450cam enzymes.

A buffered solution (50 mM Tris.HCI, pH 7.4), typically 3ml in volume, containing 10uM putidaredoxin, 2 uM putidaredoxin reductase, 1 uM cytochrome P-450cam mono-oxygenase (wild-type or mutant), 200 mM KCI, 50 ug/ml bovine liver catalase (Sigma), and 1 mM target organic compound such as cyclohexylbenzene (added as a 0.1 M stock in ethanol) was preincubated at 30°C for 5 minutes. The enzymatic reaction was initiated by adding NADH to a total

concentration of 2 mM. Further four aliquots of NADH (to increase the NADH concentration by 1mM each time) were added in intervals of 10 minutes, and 30 minutes into the incubation one aliquot of substrate (to increase the concentration by 1mM) was also added. The reaction was quenched after 60 minutes by adding 0.5 ml chloroform and vortexing the mixture. The phases were separated by centrifugation (4000 g) at 4°C. The chloroform layer was analyzed by gas chromatography.

For many substrate compounds such as cyclohexylbenzene for which not all the P-450cam-mediated oxidation products are commercially available, the chloroform extracts are evaporated to dryness under a stream of nitrogen. The residues were extracted with hexane and the oxidation products separated by high performance liquid chromatography, eluting with a hexane/isopropanol gradient. The purified products were then identified by mass spectroscopy and particularly nuclear magnetic resonance spectroscopy.

For different substrates of different solubility in the aqueous buffer solution, the amount of substrate added to the incubation mixtures varies from 0.2 mM to 4 mM final concentration. The NADH concentration can be monitored at 340 nm and, in all cases, more substrates and NADH are added during the incubation.

Using the above experimental techniques, the inventors have investigated a considerable number of organic compounds as

substrates for both the wild-type P-450cam enzyme and also the mutant version Y96A. Work has included mutants designated Y96V; Y96L; Y96f: C334A; the combined mutant F87A-Y96G-F193A and the combined active site and surface mutants of Y96A-C334A; Y96V-C334A; Y96L-C334A;Y96F-C334A;F87A-Y96G-F193A-C334A. The results for C334A and C334A-Y96A are set out in Table 1 and 2, in which structurally related molecules are grouped together.

Table 1 details the NADH consumption for oxidation of small linear, branched and cyclic hydrocarbons by the mutant Y96A-C334A. Tables 2(a) to 2(h) details the product distributions for mutant and substrate combinations where these have been elucidated to date.

The cysteine residue at position 344 can be deleted by any well known and freely available standard restriction techniques and will therefore not be described in detail herein.

**Scheme 1:**

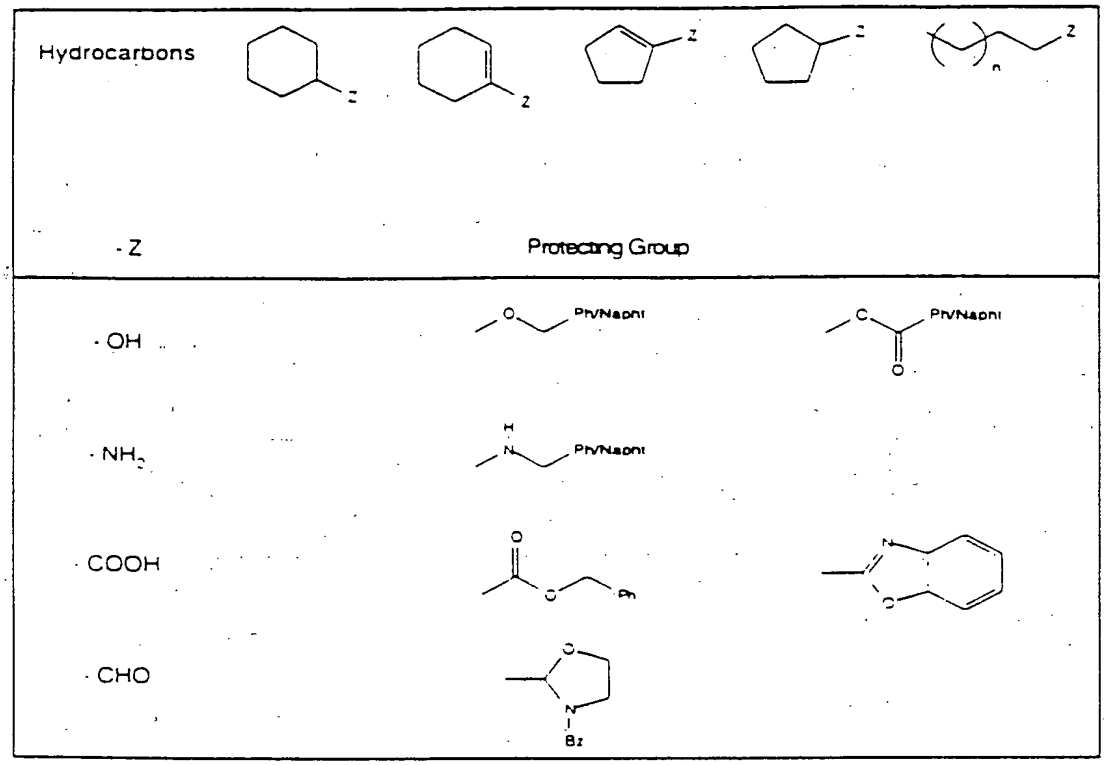
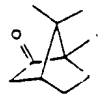
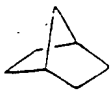
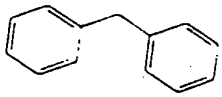

Hydrocarbons
Z
Z
Z
Z
n
Z
- Z
Protecting Group
- OH
PhNaphl
PhNaphl
- NH2
H
N
PhNaphl
- COOH
O
O
Ph
N
O
- CHO
O
N
Bz

**Table 1:**

| | | $K_{300}$ (μM)[a] | |
|---|---|---|---|
| | | WT | Y96A |
|  | 1 | 6.3 | 12 |
|  | 2 | 12 | 28 |
|  | 3 | 8.4 | 1.4 |
|  | 4 | 330 | 92 |
|  | 5 | >1500[b] | 73 |

[a] Values are the average of two independent measurements using the method of Sligar (S.G. Sligar, *Biochemistry*, **1976**, *15*, 5399 - 5406). The value of $K_{app}$ is strongly dependent on the concentration of $K^+$ in the buffer. At $[K^+]>150$ mM, $K_{app}$ for camphor is 0.6 μM for both wildtype and Y96A. Data in this table were determined at $[K^+] = 70$ mM in phosphate buffer, pH 7.4, in order to avoid salting out of substrates at higher ion concentrations.

[b] Saturation not reached.

**P450cam-substrate Interactions**

**Subgroup: 1-ring**

**Table 2(a)**

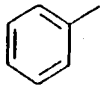
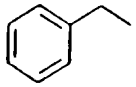
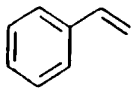
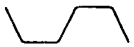
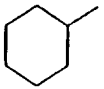
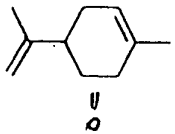
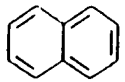
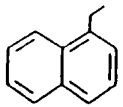
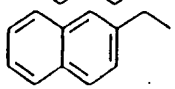
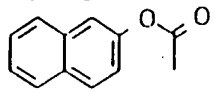
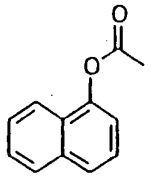
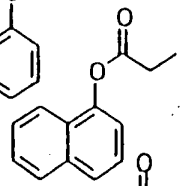
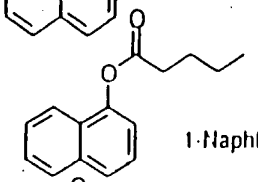
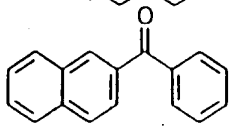
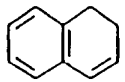
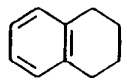
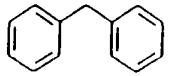
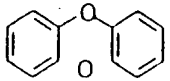
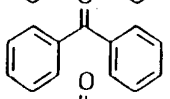
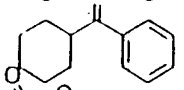
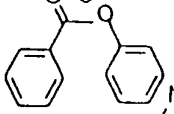
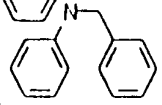
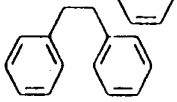
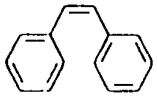
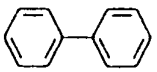
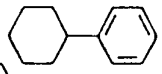
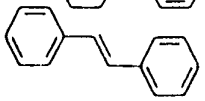
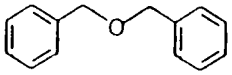
| | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
|  Benzene | - | - | - | - | | | | |
|  Toluene | - | - | 30 | 30 | | | | |
|  Ethylbenzene | - | - | 40 | 40 | | | | |
|  Styrene | - | - | 30 | 30 | | | | |
|  Cyclohexene | - | 5 | 40 | 40 | | | | |
|  1,3-Cyclohexadiene | nd | nd | nd | nd | | | | |
|  1,4-Cyclohexadiene | - | 5 | 15 | 20 | | | | |
|  Cyclohexane | - | - | 60 | 60 | | | + | |
|  Hexane | - | - | 70 | 60 | | | + | |
|  Methylcyclohexane | 50 | 50 | 100 | 70 | | | | |
|  (S)-(+)-Carvone | 10 | 60 | 10 | 80 | | | | |

Table 2(b)

P450cam-substrate interactions

| Subgroup: 2-ring, Naphthalene | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
|  Naphthalene | · | · | 15 | 20 | | | | |
|  1-Ethylnaphthalene | · | · | 5 | 20 | | | | |
|  2-Ethylnaphthalene | · | · | 10 | 20 | | | | |
|  2-Naphthylacetate | · | 5 | · | 5 | | | | |
|  1-Naphthylacetate | · | 5 | · | 5 | | | | |
|  1-Naphthylpropionate | · | 20 | 0 | 20 | | | | |
|  1-Naphthylbutyrate | · | 5 | · | 5 | | | | |
|  Naphthylphenylketone | · | 5 | · | 5 | | | | |
|  1,2-Dihydronaphthalene | 5 | 20 | 30 | 90 | | | | |
|  1,2,3,4-Tetrahydro naphthalene | 5 | 10 | 40 | 40 | | | | |

## Table 2(c)

| P450cam-substrate interactions | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| Subgroup: 2-ring, DPM | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
|  Diphenylmethane | | 5 | 45 | nd | | | + | + |
|  Diphenylether | 10 | 5 | 20 | 50 | | | | |
|  Benzophenone | | 20 | | 20 | | | | |
|  Cyclohexylphenylketone | | 30 | 60 | nd | | | | |
|  Phenylbenzoate | | 5 | | | | | | |
|  N Phenylbenzylamine | | 5 | 45 | nd | | | | |
|  Bibenzyl | | | 55 | 55 | | | | |
|  cis Stilbene | | 20 | 40 | 50 | | | | |
|  Biphenyl | | 20 | | 90 | | | | |
|  Cyclohexylbenzene | 20 | 20 | 80 | nd | | | | |
|  *trans* Stilbene | | | | | | | | |
|  Benzylether | | 5 | 55 | nd | | | | |

## Table 2(d)

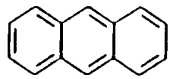
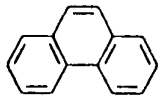
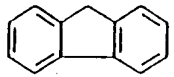
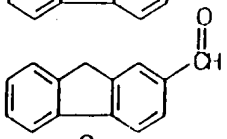
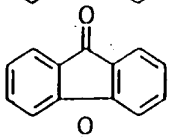
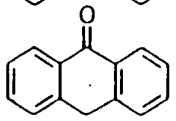
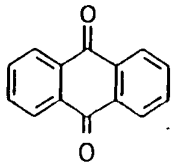
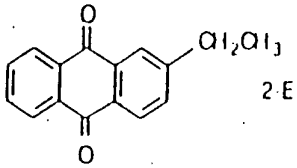
| P450cam-substrate interactions | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| Subgroup: 3-ring | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
|  Anthracene | | | | | | | | |
|  Phenanthrene | | | 20 | 20 | | | | |
|  Fluorene | | | | 50 | | | | |
|  2-Fluorencarboxaldehyde | | | | 50 | | | | |
|  9-Fluorenone | | 20 | | 5 | | | | |
|  Anthrone | | 5 | | 5 | | | | |
|  Anthraquinone | | | | | | | | |
|  2-Ethylanthraquinone | | | | | | | | |

Table 2(e)

P450cam-substrate interactions

Subgroup: 4,5-ring

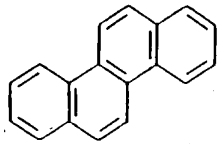
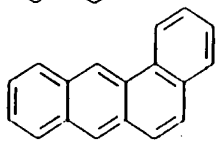
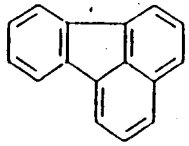
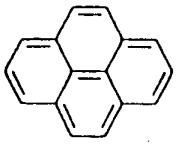
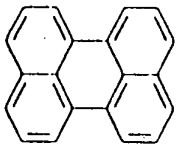
| | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
|  Chrysene | | | | | | | | |
|  1,2-Benzanthracene | | | | | | | | |
|  Fluoranthene | | 5 | 20 | 10 | | | | |
|  Pyrene* | | | | | | | | |
|  Perylene* | | | | | | | | |

Table 2(f)

P450cam-substrate interactions

Subgroup: Cyclic Alkanes

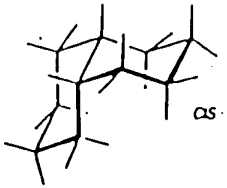
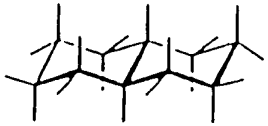
| | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|
| | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
|  *cis*-Decahydronaphthalene | nd | nd | nd | nd | | | | |
|  *trans*-Decahydronaphthalene | 20 | 10 | 90 | 70 | | | | |
|  Cyclohexane | | | 60 | 60 | | | | |
|  Methylcyclohexane | 50 | 50 | 100 | 70 | | | | |

Table 2(g)

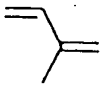
| P450cam-substrate interactions | | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|---|
| **Subgroup: n-Alkanes** | | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
| | n-Pentane | - | 5 | 55 | 40 | | | + | |
| | n-Hexane | - | - | 60 | 40 | | | + | |
| | n-Heptane | 5 | 5 | 60 | 40 | | | + | |
| | n-Octane | - | 5 | 80 | 45 | | | + | |
| | n-Nonane | - | - | 70 | 45 | | | + | |
| | n-Decane | nd | nd | nd | nd | | | | |
| | n-Undecane | nd | nd | 20 | 20 | | | | |
| | n-Dodecane | nd | nd | 5 | 5 | | | | |
| $CH_3(CH_2)_{14}CH_3$ | n-Hexadecane | - | - | - | - | | | | |
| $CH_3(CH_2)_{15}CH_3$ | n-Heptadecane | - | - | - | - | | | | |
| $CH_3(CH_2)_{11}OSO_3.Na$ | SDS | - | 20 | - | 60 | | | | |
| $CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$ | Oleic acid* | - | 10? | - | 20? | | | | |
| $[(CH_3)_2CH(CH_2)_3CH(CH_3)(CH_2)_3CH(CH_3)CH_2CH_2-]_2$ | Squalane | - | - | - | 20 | | | | |
|  | Isoprene | - | - | 10 | 10 | | | | |

Table 2(h)

P450cam-substrate Interactions

Subgroup: Camphor-like

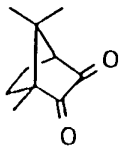
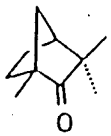
| | | Wild type | | Mutant Y96A | | Wild type | | Mutant Y96A | |
|---|---|---|---|---|---|---|---|---|---|
| | | ΔSpin high/low | Vs DTT | ΔSpin high/low | Vs DTT | NADH turnover? | GC? | NADH turnover? | GC? |
|  | (1R)-(-)-Camphorquinone | 80 | 80 | 80 | 80 | | | | |
|  | (1R)-(-)-Fenchone | 40 | 70 | 50 | 80 | | | | |
|  | Dicyclopentadiene | 50 | 80 | 90 | 90 | | | | |

Table 3

# Turnover of Small Alkanes by P450cam Mutants

All mutants listed below also contain the C334A mutation.

Turnover rate measured as NADH consumption rate (nmole NADH/nmole P450cam/s).

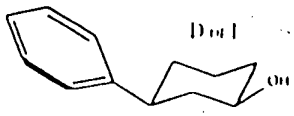
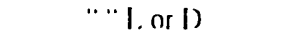
| Alkane substrate: Main chain length | Name | Wild type | Y96A |
|---|---|---|---|
| C4 | *n*-butane | - | - |
| C4 | 2-methyl butane | background | 4.6 |
| C4 | 2,3-dimethyl butane | background | 16.8 |
| C4 | 2,2-dimethyl butane | background | 14.0 |
| C5 | *n*-pentane | background | 5.8 |
| C5 | 2-methyl pentane | 3.8 | 11.7 |
| C5 | 3-methyl pentane | 1.3 | 14.2 |
| C5 | 2,4-dimethyl pentane | 0.2 | 12.6 |
| C5 | 2,2-dimethyl pentane | 5.2 | 12.8 |
| C5 | 2,2,4-trimethyl pentane | 0.9 | 5.3 |
| C5 | 3-ethyl pentane | background | 16.2 |
| C6 | *n*-hexane | background | 6.0 |
| C6 | 2-methyl hexane | background | 10.6 |
| C7 | *n*-heptane | 2.7 | 4.4 |
| C7 | 2-methyl heptane | background | 2.1 |
| C7 | 4-methyl heptane | 1.4 | 10.2 |
| C8 | *n*-octane | background | 5.8 |
| C7 | cycloheptane | 4.4 | 42.5 |

Product structures and distributions following oxidation of substrates with P450cam active site mutants.

"background" - typical background NADH oxidation rate is 0.07 nmole NADH (nmole P450cam) $^{-1}$ sec $^{-1}$

Table 4(a)

Product structure and distributions following oxidation of substrates with P450cam active site mutants. All mutants shown below also contain tl C334A mutation.

| Cyclohexylbenzene Products | | Products (%) for mutants: WT | Y96A | Y96F | Y96L | Y96V |
|---|---|---|---|---|---|---|
|  | 3-ol | 43 | 20 | 54 | 38 | 28 |
|  | 3-ol | 20 | 20 | 27 | 23 | 39 |
|  | Trans -4-ol | 25 | 15 | 6 | 23 | 10 |
|  | Cis- 4-ol | 12 | 45 | 13 | 16 | 23 |
| Total products(area/$10^5$) | | 0.8 | 7.4 | 1.1 | 10.4 | 12.5 |

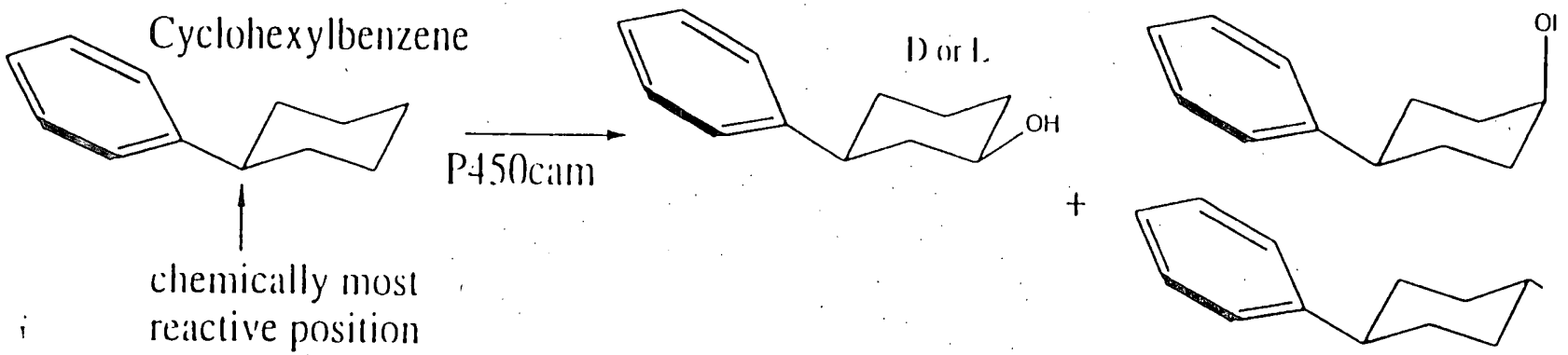
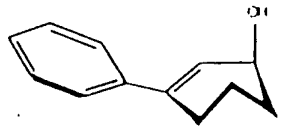


Table 4(b)

| Phenylcyclohexene Products | | Products (%) for mutants: WT | Y96A |
|---|---|---|---|
|  | 3-one (A) | 24 | 25 |
|  | 3-ol (B) | 76 | 75 |
| Total products(area/$10^6$) | | 42 | 36 |

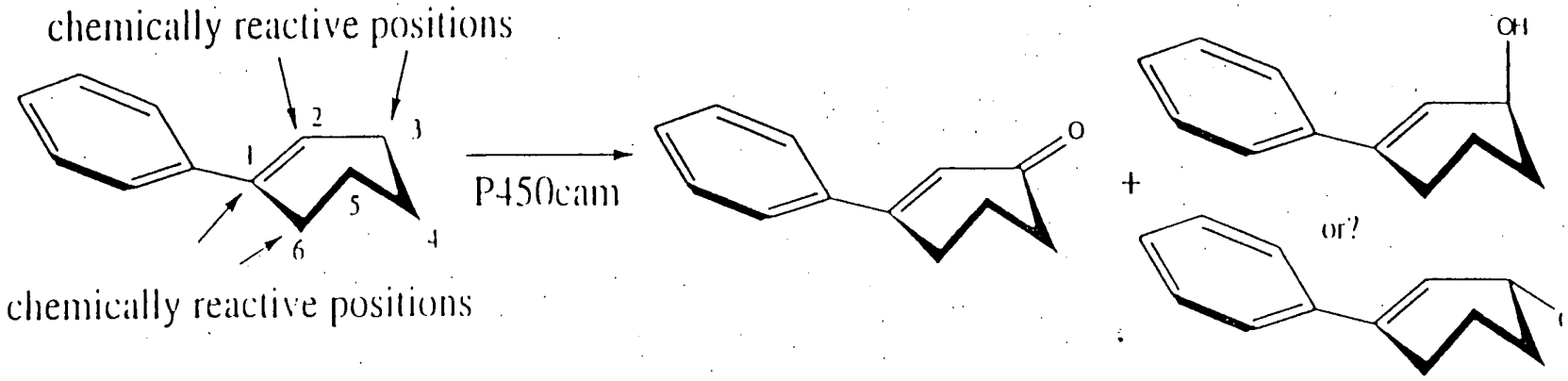
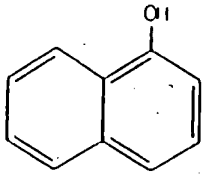
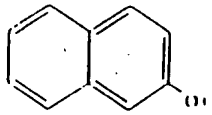


Table 4(c)

| Naphthalene Products | | Products (%) for mutants: WT | Y96A | Y96F | Y96L | Y96V | F87A-F96G-F193A |
|---|---|---|---|---|---|---|---|
|  | 1-ol | 100 | 100 | 100 | 100 | 100 | 100 |
|  | 2-ol | 0 | 0 | 0 | 0 | 0 | 0 |
| Total products (area/$10^5$) | | (0.016) | 1.1 | 2.4 | 0.7 | 1.4 | 0.1 |

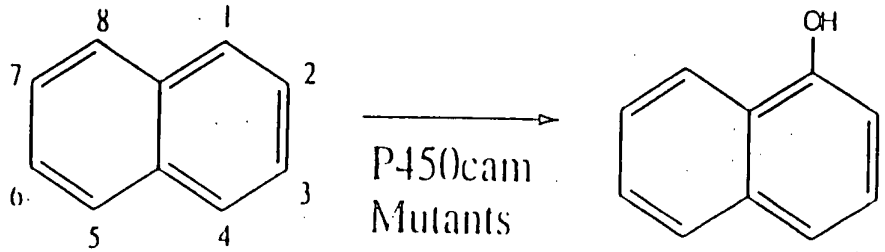
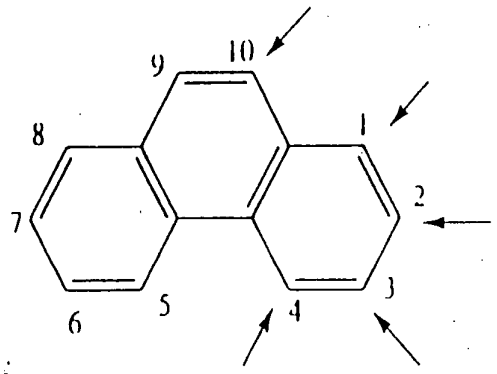


Table 4(d)

| Phenanthrene Products | Products (%) for mutants: WT | Y96A | Y96F | Y96L | Y96V | F87A-F96G-F193A |
|---|---|---|---|---|---|---|
| A | 38 | 49 | 41 | 35.5 | 41 | 27 |
| B | 15 | 23 | 31 | 41 | 38 | 41 |
| C | 12 | 13 | 5 | 9 | 11 | 3 |
| D | 35 | 15 | 23 | 14.5 | 10 | 29 |
| Total products (area/$10^6$) | 0.075 | 7.0 | 4.5 | 2.8 | 1.6 | 0.065 |

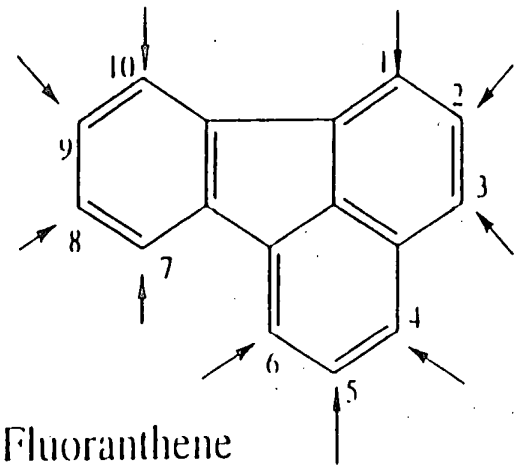
Phenanthrene




P450cam mutants → 4 hydroxylated products

Table 4(e)

| Fluoranthene Products | Products (%) for mutants: WT | Y96A | Y96F | Y96L | Y96V | F87A-F96G-F193A |
|---|---|---|---|---|---|---|
| A | 0 | 84 | - | - | - | 0 |
| B | 0 | 16 | - | - | - | 100 |
| Total products (area/$10^6$) | 0 | 2.7 | - | - | - | 0.2 |




Fluoranthene

P450cam mutants → 2 hydroxylated products

Table 4(f)

| Pyrene Products | Products (%) for mutants: WT | Y96A | Y96F | Y96L | Y96V | F87A-F96G-F193A |
|---|---|---|---|---|---|---|
| A | 0 | 40 | 43 | 23 | 30 | 33 |
| B | 0 | 43.6 | 29 | 64.5 | 55 | 40 |
| C | 0 | 5 | 12.5 | 7.9 | 12 | 20 |
| D | 0 | 11.4 | 15.5 | 4.6 | 3 | 7 |
| Total products (area/$10^6$) | 0 | 1.2 | 1.5 | 1.5 | 1.6 | 0.02 |

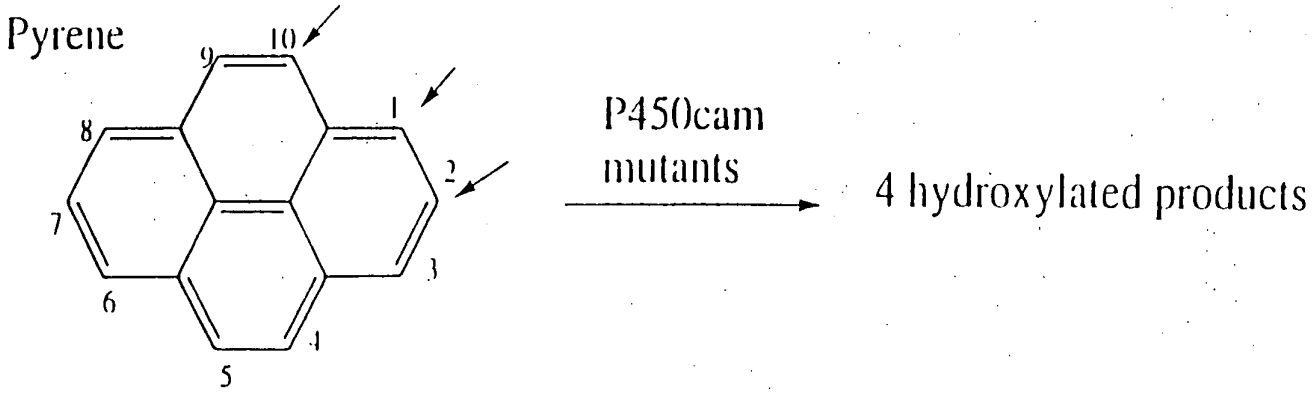
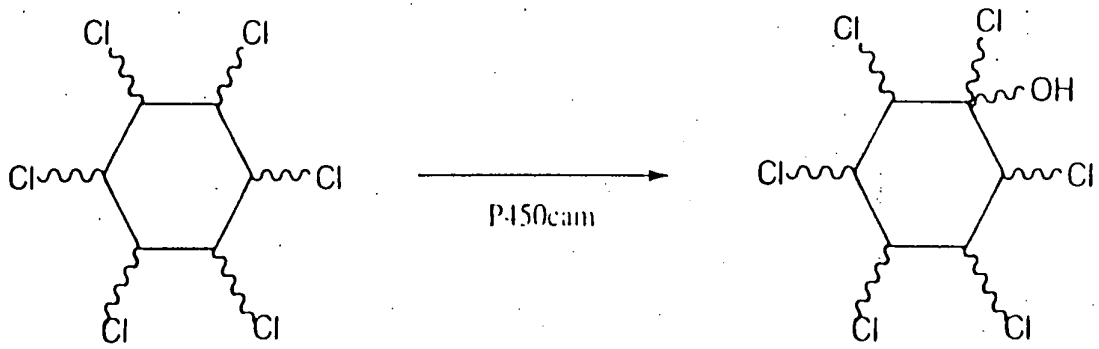


Table 4(g)

| Lindane Products (hexachlorocyclohexane) | Products (%) for mutants WT | Y96A |
|---|---|---|
| A | 100 | 100 |
| Turnover rate nmole NADH (nmoleP450) $^{-1}$s $^{-1}$ | 7.5 | 43.5 |




Hexachlorocyclohexane

Table 4(h)

| Hexane Products | Products (%) for mutants: | |
|---|---|---|
| | Y96F | Y96A |
| 2-hexanone | 10 | 15 |
| 3-hexanone | 16 | 28 |
| 2-hexanol | 24 | 26 |
| 3-hexanol | 50 | 32 |
| Relative activity (WT = 1) | 18.2 | 25.5 |

| 2-Methyl hexane Products | Products (%) for mutants: | |
|---|---|---|
| | Y96F | Y96A |
| 2-methyl-2-hexanol | 72 | 74 |
| 5-methyl-2-hexanone | 16 | 14 |
| 2-methyl-3-hexanol | 7 | 4 |
| 5-methyl-2-hexanol | 5 | 8 |
| Relative activity (WT = 1) | 2.3 | 2.6 |

**REFERENCES**


1. "Cytochroe P-450: Structure, Mechanism, and Biochemistry", ed. P R Ortiz de Montellano, Plenum Press, New York,1986.

2. T L Poulos, B C Finzel and A J Howard, J. Mol. Biol., t987, 195, 687-700.

3. C A Hasemann, K G Ravichandran, J A Peterson, and J Deisenhofer, J. Mol. Biol., 1994, 2-)6, 1169-1185.

4. K G Ravichandran, S S Boddupalli, C A Hasemann, J A Peterson, and J Deisenhofer, Science, 1993, 261, 731-736.

5. B P Unger, I C Gunsalus, and S G Sligar, J. Biol. Chem., 1986,261,1158-1163; J S Miles, A W Munro, B N Rospendowski, W E Smith, J McKnight, and A J Thomson, Biochem. J., 1992, 288, 503-509; T H Richardson, M J Hsu, T Kronbach, H J Bames, G Chan, M R Waterman, B Kemper, and E F Johnson, Arch. Biochem. Biophys., 1993, 300, 510-516; S S Boddupalli, T Oster, R W Estabrook, and J A Peterson, J. Biol. Chem., 1992, 267, 10375-10380; H Li K Darish and T L Poulos. J. Biol. Chem., 1991, 266, 11909-11914.

6. 1 C Gunsalus and G C Wagner, Methods Enzymol., 1978, 52, 166-188.

7. M Haniu, L G Armes, K T Yasunobu, B A Shastry, and I C Gunsalus. Biol. Chem., 1982, 257, 12664-12671.

8. S G Sligar, D Filipovic, and P S Stayton, Methods Enzymol., 1991, 206, 31-49.

9. P J. Loida and S G Sligar, Biochemistrv, 1993, 32, 11530-11538.

10. P J Loida and S G Sligar, Protein Eng., 1993, 6, 207-212.

11. W M. Atkins and S G Sligar, J. Am. Chem. Soc., 1989, 111, 2715-2717.

12. S F Tuck, S Graham-Lorence, J A Peterson, and P R Ortiz de Montellano, J.Biol. Chem., 1993, 268, 269-275.

13. C Di Prime, G Hui Bin Hoa, P. Douzou, and S Sligar, J. Biol. Chem., 1990, 265, 5361-5363.

14. W M Atkins and S G Sligar, J. Biol. Chem., 1988, 263, 18842-18849.

15. W M Atkins and S G Sligar, Biochemistry, 1990, 29, 1271-1275.

16. J D Lipscomb, J E Harrison, K M Dus, and I C Gunsalus, Biochem. Biophys. Res. Conunun., 1978, 83, 771-778.

17. P J Goodford, J. Med. Chem., 1985, 28, 849-857.

18. Quanta 4.0, Molecular Simulations Inc., 16 New England Executive Park, Burlington, MA 01803-5297.

19. J A Fruetet, J R Collins, D L Camper, G H Loew, and P R Ortiz de Montallano, J. Am. Chem. Soc., 1992, 114, 6987-6993.

20. Y Watanabe and Y Ishimura, J. Am. Chem. Sec., 1989, 111, 410-41 1.

21. J P Jones, W F Trager, and T J Carlson, J. Am. Chem. Soc., 1993, 115, 381-387.

22. "Biotransformation in Preparative Organic Chemistry" H G Davis, R H Green, D R Kelly, and S M Roberts, Academic Press, London, 1989, Page 169 ff.

23. J E Baldwin J M Blackburn, R J Heath, and J D Sutherland, Bioorg. Med. Chem. Letts. 1992, 2, 663-668.

24. M J Zoller and M Smith, Nucleic Acids Res., 1982, 10, 6487-6500.

25. T A Kunkel, Proc. Natl. Acad. Sci., USA 1985, 82, 488-492.

26. S G Sligar and I C Gunsalus, Proc. Natl. Acad. Sci., USA, 1976, 73, 1078-1082.

**CLAIMS**

1. A mutant of the mono-oxygenase cytochrome P-450cam in which the cysteine residue at position 334 is removed.

2. A mutant as claimed in claim 1 in which the removal is by the substitution of another amino acid except cysteine for the cysteine residue.

3. A mutant as claimed in claim 1 in which the removal is by deletion of the entire cysteine 334 residue from the enzyme.

4. A mutant as claimed in any of the preceding claims in which the tyrosine residue at position 96 in the mutant is replaced by any other amino acid except tyrosine.

5. A mutant as claimed in either of claims 1, 2 or 4 in which the amino acid is selected from any one of the following:-

alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan, tyrosine and valine.

6. A mutant as claimed in any of the preceding claims in which the amino acid residue at one or more of the positions 87, 98, 101, 185, 193, 244, 247, 295, 297, 395 and 396 is replaced by another amino acid residue.

7. A mutant of the mono-oxygenase cytochrome P-450cam substantially as hereinbefore described with reference to the accompanying drawings and/or examples.

The Patent Office

37

**Application No:** GB 9622819.2 **Examiner:** Colin Sherrington
**Claims searched:** 1 to 7 **Date of search:** 6 December 1996

## Patents Act 1977
## Search Report under Section 17

**Databases searched:**

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.O): C3H(HB7M)

Int Cl (Ed.6): C12N 9/02,15/53

Other: ONLINE: WPI,CLAIMS,DIALOG/BIOTECH

**Documents considered to be relevant:**

| Category | Identity of document and relevant passage | Relevant to claims |
|---|---|---|
| E, A | GB 2294692 A (BRITISH GAS PLC) -whole document | 4 to 7 |
| A | J.Biological Chemistry 1988,263(35),18842-18849 -William M.Atkins *et al.* "The Roles of Active Site Hydrogen Bonding in Cytochrome P-450cam as Revealed by Site-directed Mutagenesis" | 4 |
| A | J.Amer.Chem.Soc. 1989,111,2715-2717 -William M.Atkins *et al.* "Molecular Recognition in Cytochrome P-450: Alteration of Regioselective Alkane Hydroxylation via Protein Engineering" | 4 |
| A | J.Biological Chemistry 1990,265(10),5361-5363 -Carmelo Di Primo *et al.* "Mutagenesis of a Single Hydrogen Bond in Cytochrome P-450 Alters Cation Binding and Heme Solvation" | 4 |

X Document indicating lack of novelty or inventive step
Y Document indicating lack of inventive step if combined with one or more other documents of same category.
& Member of the same patent family
A Document indicating technological background and/or state of the art.
P Document published on or after the declared priority date but before the filing date of this invention.
E Patent document published on or after, but with priority date earlier than, the filing date of this application.